ORIGINAL ARTICLE

Endothelin-1 Activates MAPKs and Modulates Cell Cycle Proteins in OKP Cells

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Background/Purpose: The signaling mechanisms through which endothelin (ET)-1 induces hyperplasia of the renal tubular epithelium are largely unknown.

Methods: These mechanisms were explored using ET_B–overexpressing opossum kidney (OKP) cells as a model system.

Results: ET-1 (10 nM) induced a 10-fold increase in *c-jun* mRNA abundance within 30 minutes and an 8-fold increase in extracellular signal-regulated kinase (ERK)1/2 activity within 5–10 minutes in these cells. ERK1/2 phosphorylation in response to ET-1 was suppressed by ET_B-receptor blockade or by treatment with an MAPK kinase (MEK) inhibitor. MEK1/2 activity increased 8-fold within 5 minutes of ET-1 treatment. Additionally 2-fold increases in cyclin D1 expression and retinoblastoma (RB) gene product phosphorylation were observed within 4 hours of treatment.

Conclusion: Binding of ET-1 to the ET_B receptor of ET_B–overexpressing OKP cells is proposed to signal proliferation of these cells through rapid activation of mitogen-activated protein kinases, increased *c-jun* expression, modulation of cyclin D1 activity, and increased RB phosphorylation. [*J Formos Med Assoc* 2007;106(4):273–280]

Key Words: cell cycle proteins, endothelin-1, immediate early genes, mitogen-activated protein kinases, OKP cells

In response to certain physiologic signals or pathologic processes, the renal tubular epithelium undergoes periods of rapid growth involving both hyperplasia and hypertrophy.[1] In addition to epidermal growth factor and angiotensin II, the endothelins (ETs) are now recognized to function importantly as growth-promoting factors for the renal tubular epithelium.[2,3] In renal tissue, the ETs serve as both paracrine and autocrine growth factors.[3] Three isoforms of ETs, termed as ET-1, ET-2 and ET-3, have been identified that interact with two receptors, termed as ET_A and ET_B. Both receptors are present in the kidney.[4,5]

The signal transduction pathways involved in renal growth regulation are relatively complex.[1,2,6] These pathways have been characterized for renal mesangial cells but not for renal tubuloepithelial cells. ET-1 stimulates renal mesangial cell proliferation through several pathways, prominent among which are those involving the mitogen-activated protein kinases (MAPKs).[7] ET-1 binds to the ET_A receptor, signals the activation of extracellular signal-regulated kinase (ERK)1/2, and modulates expression of the immediate early genes (*c-fos*, *c-jun*) to induce proliferation of renal mesangial cells.[7]

Following a mitotic signal, cells undergo a finely regulated series of changes in status that

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together comprise the cell cycle. Progression from G_1 into S phase is determined at the restriction point and is governed by the activity of retinoblastoma (RB), the product of the RB gene. In its hypophosphorylated state, RB is active and serves to block progression to S phase. Upon phosphorylation, RB is inactivated such that progression into S phase occurs.[1] The cell cycle is ultimately controlled by cell cycle-regulatory proteins. Transitions between each phase of the cell cycle are positively regulated by the kinase activity of a distinct holoenzyme composed of two subunits: the cyclins and their partners, the cyclin-dependent kinases (CDKs). CDK inhibitors negatively regulate cell cycle progression, and subsequently arrest the cell cycle, by inhibiting formation of CDK complexes.[8] In rat mesangial cells, cyclin D1-CDK activity and the phosphorylation of RB mediate the mitogenic response to ET-1.[9]

Epithelial and endothelial cells often utilize similar signaling systems during the process of tubulation. However, it is unclear whether growth factors such as endothelin promote hyperplasia of the renal mesangium and tubuloepithelium through comparable mechanisms. Cultured opossum kidney (OKP) cells, which are subcloned from an opossum kidney cell line, exhibit many characteristics of proximal tubuloepithelial cells and therefore represent a useful *in vitro* model of these cells.[10] For example, ET-1 acutely increases Na/H antiporter activity in OKP cells stably transfected with ET_B receptor cDNA and induces these ET_B–overexpressing cells to proliferate.[11,12] The present report describes the effects of ET-1 on the expression of immediate early genes, the activation state of MAPKs, and the disposition of cell cycle regulatory proteins in ET_B–overexpressing OKP cells.

Materials and Methods

Materials

Culture media, fetal bovine serum (FBS), and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA). Culture flasks and plates were purchased from Corning (Corning, NY, USA). Blocking reagent, alkaline phosphatase-conjugated antidigoxigenin antibody, CSPD (chemiluminescent substrate alkaline phosphatase detection), and digoxigenin-UTP (uridine triphosphate) were purchased from Boehringer Mannheim (Mannheim, Germany). The pGEM-dT vector was purchased from Promega (Madison, WI, USA). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate were obtained from Sigma (St. Louis, MO, USA). Bicinchoninic acid (BCA) reagents were obtained from Pierce (Rockford, IL, USA). Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-MEK1/2, and anti-MEK1/2 were purchased from New England Biolabs (Beverly, MA, USA). BQ123, BQ788, and PD98059 were purchased from Research Biochemicals International (Natick, MA, USA). Anti-cyclin D1, anti-CDK4, anti-RB, anti-Ser795-phosphorylated RB, and anti-p27 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin was obtained from Chemicon (Tamecula, CA, USA). PVDF membranes were obtained from Millipore (Bedford, MA, USA). Enhanced chemiluminescence (ECL) kits were obtained from Amersham (Arlington Heights, IL, USA).

Cell culture

OKP cells overexpressing the ET_B receptor (OKP ETB6 clone) were provided by Dr Robert Alpern.[11] The cells were propagated in high glucose (450 mg/dL) Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). For experimentation, cells were grown in low glucose (100 mg/dL) DMEM. When confluent, cells were rendered quiescent by incubation for 48 hours in serum-free medium.

RNA preparation and analysis

Total cellular RNA was extracted with guanidinium thiocyanate/phenol/chloroform and isolated by ethanol precipitation.[13] Twenty microgram of total RNA were size-fractionated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes. RNA blots were hybridized

overnight at 65°C with antisense RNA probe in hybridization solution composed of 50% formamide, 5X SSCP (1X SSCP is 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, and 1 mM EDTA, pH 7.2), 0.1% sodium lauryl sarcosine, 2% sodium dodecyl sulfate (SDS), and 5% blocking reagent. Blots were then washed in 0.1X SSCP and 0.1% SDS at 65°C and developed using alkaline phosphatase-conjugated antidigoxigenin antibody and CSPD according to the manufacturer's directions.

Probe synthesis

To synthesize the *c-jun* riboprobe for Northern blot hybridization, a cDNA fragment of rat *c-jun* was first amplified by the reverse transcription polymerase chain reaction from glomerular RNA of Wistar rats using the following specific primer pair: rat *c-jun*, upstream, 5′-TTCTGAAGCAGAGCATGACC-3′ and downstream, 5′-TTGAAGTTGCTGAGGTTGGC-3′ (GeneBank accession no. X17163). The amplified products were eluted from acrylamide gels and subcloned into the pGEM-dT vector. The antisense RNA probe was transcribed using phage polymerase in the presence of digoxigenin-UTP from subcloned templates.[14]

Protein extraction for Western blotting

Cultured cells were washed three times in phosphate-buffered saline (PBS), scraped into RIPA buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (TRIS) HCl, pH 7.4, 2.5 mM EDTA, 5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.5 mM dithiothreitol, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 μg/mL aprotinin], incubated at 4°C for 45 minutes, and centrifuged at 10,000*g* for 15 minutes.

Supernatants were diluted with RIPA buffer, and total protein contents were determined with a BCA protein assay.

Measurement of ERK and MEK activities

The Western blotting analysis of ERK phosphorylation was performed with phospho-specific antibodies according to the manufacturer's protocol. Briefly, OKP cells were stimulated with ET-1 at different time points. Inhibitors (BQ788, BQ123, and PD98059) were added to incubations 30 minutes prior to addition of ET-1.[15,16] Cell lysates (50 μg protein) were subjected to SDS-PAGE (10%) and proteins were then transferred to PVDF membrane. For immunodetection, the membranes were probed with phospho-specific ERK antibody (1:1000) followed by incubation with peroxidase-conjugated secondary antibody (1:4000). Bands were visualized with ECL system. MEK and p38 MAPK activities were measured by a similar method.

Western blot analysis of cell cycle proteins

For Western blot analysis, 50 μg of protein extract were treated for 5 minutes at 100°C, and denatured proteins were separated by 10% SDS-PAGE. After electrophoresis, electroblotting onto PVDF membranes was performed, and the blots were stained with Coomassie blue to ensure complete protein transfer. Blots were incubated in 1X PBS containing 0.1% Tween-20 and 5% nonfat milk to prevent nonspecific antibody binding. Blots were then incubated for 2 hours at room temperature with antibodies for cyclin D1, CDK4, RB, Ser795-phosphorylated RB, or p27. Blots were washed in 0.1% Tween-20 in PBS for 15 minutes, re-washed at least twice for 5 minutes, and incubated with a 1:5000 dilution of horseradish peroxidase-labeled anti-mouse IgG in 5% nonfat milk and 0.05% Tween-20 in PBS for 1 hour. Western blots were developed by the ECL system.

Results

ET-1 increases c-jun mRNA abundance

As shown in Figure 1A, treatment with ET-1 at 10 nM ET-1 increased *c-jun* mRNA abundance in ET_B–overexpressing OKP cells. This effect was detectable at 15 minutes and maximal at 30 minutes, and an optimal increase in message of approximately 10-fold was observed. After 30 minutes of treatment, *c-jun* mRNA abundance decreased to

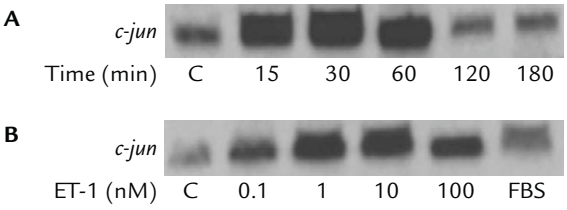

Figure 1. Endothelin-1 (ET-1) increases *c-jun* mRNA abundance in ET_B–overexpressing OKP cells. (A) Time course with 10 nM ET-1. (B) Dose response at 30 minutes. Results shown are typical of three separate experiments. C = control; FBS = fetal bovine serum.

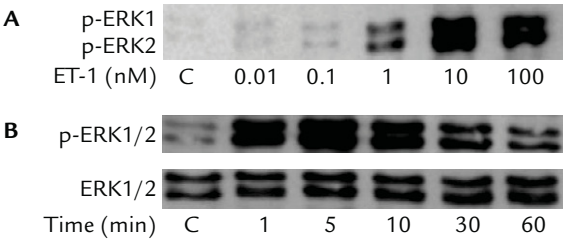

Figure 2. Endothelin-1 (ET-1) increases extracellular signal-regulated kinase (ERK1/2) phosphorylation in ET_B–overexpressing OKP cells. (A) Dose response at 10 minutes. (B) Time course with 10 nM ET-1. Results shown are typical of three separate experiments. C = control.

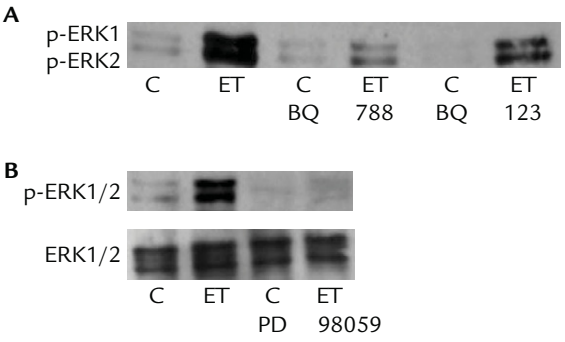

Figure 3. Endothelin-1 (ET-1) induced-ERK1/2 phosphorylation is mediated by ET_B receptor and MAPK kinase (MEK). (A) Cells were treated with 10 nM ET in absence or presence of BQ788, an ET_B-selective blocker, or BQ123, an ET_A-selective blocker. (B) Cells were treated with 10 nM ET in absence or presence of PD98059, an MEK inhibitor. Results shown are typical of three separate experiments. C = control.

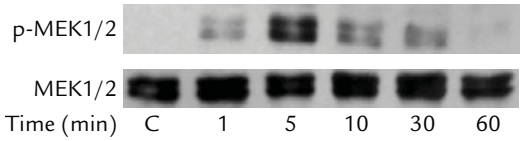

Figure 4. Endothelin-1 (ET-1) increases MAPK kinase (MEK1/2) phosphorylation in ET_B–overexpressing OKP cells. Results shown are typical of three separate experiments. C = control.

approach basal values. The dose-response relationship for this effect of ET-1 is shown in Figure 1B. Threshold and peak responses were 1 and 10 nM ET-1, respectively.

MAPK activation in response to ET-1

ET-1 treatment enhanced ERK1/2 activity in a dose-dependent manner with threshold and peak responses of 1 and 10 nM ET-1, respectively, observed (Figure 2A). The maximal stimulation of ERK phosphorylation by ET-1 was approximately 8-fold as compared with that in control preparations. The time dependence for ET-1-dependent ERK1/2 phosphorylation is illustrated in Figure 2B. Phosphorylation of ERK1/2 was increased within 1 minute and was maximal at 5–10 minutes. In contrast, the activity of p38 MAPK was not enhanced by ET-1 in these cells (data not shown).

Effects of BQ788, BQ123, and PD98059 on ET-1-stimulated ERK1/2 phosphorylation

Because these studies were performed with ET_B receptor–overexpressing cells, it was probable that observed effects of ET-1 were mediated by the ET_B receptor. Antagonists selective for the ET_A or ET_B receptor were therefore employed to validate this assumption. ET-1-induced ERK1/2 phosphorylation was significantly reduced in the presence of 10^{-7} M BQ788, an ET_B-selective blocker, but was unaffected in the presence of 10^{-7} M BQ123, an ET_A-selective blocker (Figure 3A). ERK1/2 phosphorylation in response to ET-1 was also reduced significantly in the presence of 10^{-5} M PD98059, an inhibitor of MEK (Figure 3B).

MEK1/2 phosphorylation in response to ET-1

As shown in Figure 4, ET-1 (10 nM) treatment increased MEK1/2 activity in ET_B–overexpressing OKP cells. An increase in phosphorylation of MEK1/2 was observed within 1 minute and was maximal at 5 minutes. The optimal stimulation of phosphorylation was approximately 8-fold. After 5 minutes, MEK1/2 phosphorylation was observed to decrease to approach basal values.

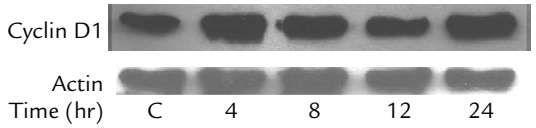

Figure 5. Endothelin-1 (ET-1) increases cyclin D1 expression in ET_B–overexpressing OKP cells. Results shown are typical of three separate experiments. C = control.

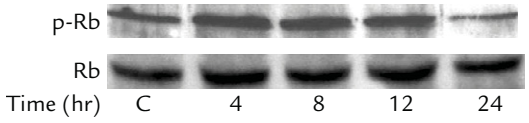

Figure 6. Endothelin-1 (ET-1) increases retinoblastoma gene product (RB) phosphorylation in ET_B–overexpressing OKP cells. Results shown are typical of three separate experiments. C = control; pRB = hyperphosphorylated form of RB.

Disposition of cell cycle proteins in ET-1-treated ET_B–overexpressing OKP cells

Treatment with 10 nM ET-1 resulted in a 2-fold increase in cyclin D1 expression in these cells, with maximal stimulation observed at 4 hours. In the lane of 12 hours, there is very minimal induction (Figure 5). However, expression of CDK4 was not enhanced under these conditions (data not shown). As shown in Figure 6, 10 nM ET-1 also induced a 2-fold increase in RB phosphorylation, with maximal effects also observed at 4 hours. In the lane of 24 hours, there is decrease in the phosphorylation levels of RB. However, ET-1 did not alter the expression of p27 in these cells (data not shown).

Discussion

Although ET-1 has been shown to induce hyperplasia in ET_B–overexpressing OKP cells,[12] the signaling mechanisms through which hyperplasia is induced were previously unknown. The present study provides evidence that ET-1 signals the activation of MEK1/2 and ERK1/2, an increase in immediate early gene (*c-jun*) expression, the modulation of cyclin D1, and an increase in RB phosphorylation in these cells. These events are likely to be relevant to those in normal renal tubular epithelial cells. The effects of ET-1 at the renal tubule are thought to be mediated mainly by the ET_B receptor whereas those at the renal glomerular mesangium are known to be mediated by the ET_A receptor. Endothelin has been shown to bind to S1 proximal tubule cells, with ET-1 and ET-3 binding occurring at similar magnitudes, consistent with the presence of the ET_B receptor in these cells.[17] Findings of the present study with ET_B–overexpressing OKP cells are consistent with involvement of the ET_B receptor in the mitogenic effects of ET-1 at the renal tubular epithelium. In particular, ET-1-dependent ERK1/2 activation in the OKP cells was reduced by ET_B-selective receptor blockade.

The protein products of the immediate early genes *c-fos* and *c-jun* function cooperatively as the components of the mammalian transcription factor activator protein (AP)-1 to induce cell proliferation.[18] Consistent with involvement of the immediate early genes in the actions of ET-1 in ET_B–overexpressing OKP cells, ET-1 was observed to increase *c-jun* mRNA abundance to maximal degrees within 30 minutes. Endothelin increases *c-fos* mRNA and *c-jun* mRNA abundance in rat mesangial cells to similar degrees and over comparable time periods.[19]

MAPKs can be divided into three broad families on the basis of sequence similarity, upstream activators, and substrate specificity.[20] The classic extracellular signal-regulated kinases (ERKs: ERK1 and ERK2) are recognized to function during growth factor-related signaling, whereas the jun NH_2-terminal kinase (JNK) and p38 families are known to be activated during cellular responses to stress and inflammation.[20] ET-1 (10 nM) was found to activate ERK1/2, but not p38, in ET_B–overexpressing OKP cells. This selective activation of ERK is not surprising in that ET-1, a growth factor, is known to signal ERK activation in other tissues.[7,21] ET-1 rapidly activates ERK1/2 in renal mesangial cells but higher concentrations of the factor (100 nM) are required.[22,23] The high concentration of ET_B receptors in the cells selected for the present study may explain this discrepancy. Alternatively, the inconsistency may be attributable to differences in receptor and/or cell type.

MEKs are upstream activators of MAPK. Accordingly, ET-1 was found to promote activation of MEK1/2 in ET_B–overexpressing OKP cells, with a maximal response occurring within 5 minutes. Comparable signaling of MEK activation is also observed in rat glomerular mesangial cells in response to ET-1.[24]

The proteins cyclin D1 and CDK4 regulate the cell cycle at G1 in a positive manner.[8] ET-1 signaled the activation of cyclin D1 and phosphorylation of RB in ET_B–overexpressing OKP cells without affecting the activity of CDK4 or p27. These findings differ from those in rat mesangial cells where ET-1 was found to increase both cyclin D1 expression and CDK4 activity via the ET_A receptor.[9] These effects are regulated by the expression of cyclin D1, p16, p21, and phosphorylatable form of RB.[9] It is clear, therefore, that the early mitogenic effects of ET-1 in renal tubuloepithelial cells are similar, but not identical, to those in renal mesangial cells. Additionally, the mitogenic actions of endothelin in rat mesangial cells are proposed to involve stimulations of phospholipase C activity, Na^+/H^+ exchange, and *c-fos* expression.[25] Whether such signaling events are required for ET-1-dependent induction of renal tubular epithelial hyperplasia is unclear.

The ET-1-dependent signaling events identified in the present study may operate during development of renal failure. Progressive renal failure results from a triad of glomerulosclerosis, tubulointerstitial fibrosis, and vascular sclerosis.[26,27] After a loss of functioning nephrons, the remaining glomeruli and tubules become hypertrophic through the action of various growth factors and/or vasoactive substances such as endothelin.[27] These reactions ultimately result in glomerulosclerosis and tubulointerstitial fibrosis.[2,27] A close association exists between the progression of chronic renal disease and structural derangements of the tubuloepithelial compartment.[28] Renal tubule cell hyperplasia and hypertrophy are both regarded as antecedents of tubulointerstitial fibrosis.[2] It is of particular interest that activation of the ERK pathway has been found to precede tubular proliferation in the obstructed rat kidney.[29]

Pentoxifylline inhibits platelet derived growth factor-stimulated mesangial cell proliferation through attenuated cyclin D1 expression and decreased RB phosphorylation.[30] Pentoxifylline and dipyridamole have renoprotective effect. Recently, we found that pentoxifylline and dipyridamole inhibited OKP cell proliferation.[31] Dipyridamole inhibits platelet derived growth factor-stimulated human peritoneal mesothelial cell proliferation through attenuated extracellular signal-regulated protein kinase activity, preservation of p27, and decreased RB phosphorylation.[32]

Endothelin-1 transgenic mice develop glomerulonephrosis, interstitial fibrosis, and renal cysts but not hypertension.[33] In this study, we demonstrated that ET_B-selective blocker inhibited ET-1 induced MAPK activation, which was the underlying signaling mechanism of cell proliferation. Renal protection of ET antagonists was mediated by both glomerular and tubular action. Bosentan, a nonselective ET antagonist, attenuates renal injury and prolongs survival in rats with remnant kidney.[34] Bosentan has been used for essential hypertension and pulmonary hypertension.[35,36]

ERKs mediate cellular functions other than cell proliferation. In OKP cells, ET-1 acutely increased Na/H exchanger-3 (NHE-3) activity through Ca-dependent pathway and tyrosine kinase pathway.[11,37] Acid incubation increases NHE-3 mRNA abundance in OKP cells.[38] Acid-induced activation of NHE3 is mediated by c-SRC and ERK.[39]

In conclusion, ET-1 was observed to interact with the ET_B receptor to activate both MEK1/2 and ERK1/2, to increase expression of *c-jun*, to alter the disposition of cyclin D1, and to increase RB phosphorylation in these cells. These signals are therefore proposed to mediate the proliferative effects of the growth factor on renal tubuloepithelial cells.

Acknowledgments

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References

1. Preisig PA, Franch HA. Renal epithelial hyperplasia and hypertrophy. *Semin Nephrol* 1995;15:327–40.
2. Wolf G. Cellular mechanisms of tubule hypertrophy and hyperplasia in renal injury. *Miner Electrol Metab* 1995;21: 303–16.
3. Kohan DE. Endothelins in the normal and diseased kidney. *Am J Kidney Dis* 1997;29:2–26.
4. Arai H, Hori S, Aramori I, et al. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990;348: 730–2.
5. Sakurai T, Yanagisawa M, Takuwa Y, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 1990;348:732–5.
6. Schocklmann HO, Lang S, Sterzel RB. Regulation of mesangial cell proliferation. *Kidney Int* 1999;56:1199–207.
7. Sorokin A, Kohan DE. Physiology and pathology of endothelin-1 in renal mesangium. *Am J Physiol-Renal Physiol* 2003;285:F579–89.
8. Shankland SJ, Wolf G. Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. *Am J Physiol-Renal Physiol* 2000;278:F515–29.
9. Terada Y, Inoshita S, Nakashima O, et al. Cyclin D1, p16, and retinoblastoma gene regulate mitogenic signaling of endothelin in rat mesangial cells. *Kidney Int* 1998;53: 76–83.
10. Cole JA, Forte LR, Krause WJ, et al. Clonal sublines that are morphologically and functionally distinct from parent OK cells. *Am J Physiol-Renal Physiol* 1989;256:F672–9.
11. Chu TS, Peng Y, Cano A, et al. Endothelin$_B$ receptor activates NHE-3 by a Ca^{2+}-dependent pathway in OKP cells. *J Clin Invest* 1996;97:1454–62.
12. Chu TS, Cano A, Yanagisawa M, et al. TGF-β and herbimycin A convert endothelin-1-induced hyperplasia into hypertrophy in renal tubuloepithelial cells. *J Am Soc Nephrol* 1995; 6:765. [Abstract]
13. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–9.
14. Chen YM, Chiang WC, Lin SL, et al. Dual regulation of tumor necrosis factor-α-induced CCL2/monocyte chemoattractant protein-1 expression in vascular smooth muscle cells by nuclear factor-κB and activator protein-1: modulation by type III phosphodiesterase inhibition. *J Pharmacol Exp Ther* 2004;309:978–86.
15. Chu TS, Wu KD, Wu MS, et al. Endothelin-1 chronically inhibits Na/H exchanger-3 in ET$_B$-overexpressing OKP cells. *Biochem Biophys Res Com* 2000;271:807–11.
16. Alessi DR, Cuenda A, Cohen P, et al. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* 1995;270; 274:89–94.
17. Takemoto F, Uchida S, Ogata E, et al. Endothelin-1 and endothelin-3 binding on rat nephrons. *Am J Physiol-Renal Physiol* 1993;264:F827–32.
18. Travali S, Koniecki J, Petralia JS, et al. Oncogenes in growth and development. *FASEB J* 1990;4:3209–14.
19. Simonson MS, Jones JM, Dunn MJ. Cytosolic and nuclear signaling by endothelin peptides: mesangial response to glomerular injury. *Kidney Int* 1992;41:542–5.
20. Tian W, Zhang Z, Cohen DM. MAPK signaling and the kidney. *Am J Physiol-Renal Physiol* 2000;279:F593–604.
21. Yoshizumi M, Kim S, Kagami S, et al. Effect of endothelin-1 (1-31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells. *Br J Pharmacol* 1998;125:1019–27.
22. Wang Y, Simonson MS, Pouysségur J, et al Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. *Biochem J* 1992;287:589–94.
23. Wang Y, Pouysségur J, Dunn MJ. Endothelin stimulates mitogen-activated protein kinase activity in mesangial cells through ET$_A$. *J Am Soc Nephrol* 1994;5:1074–80.
24. Schramek H, Sorokin A, Watson RD, et al. Differential long-term regulation of MEK and of p42 MAPK in rat glomerular mesangial cells. *Am J Physiol-Cell Physiol* 1996;270:C40–8.
25. Simonson MS, Wann S, Mene P, et al. Endothelin stimulates phospholipase C, Na^+/H^+exchange, *c-fos* expression, and mitogenesis in rat mesangial cells. *J Clin Invest* 1989;83: 708–12.
26. Becker GJ, Hewitson TD. The role of tubulointerstitial injury in chronic renal failure. *Curr Opin Nephrol Hypertens* 2000;9:133–8.
27. Brenner BM. Nephron adaptation to renal injury or ablation. *Am J Physiol-Renal Physiol* 1985;249:F324–37.
28. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 1992; 20:1–17.
29. Masaki T, Foti R, Hill PA, et al. Activation of the ERK pathway precedes tubular proliferation in the obstructed rat kidney. *Kidney Int* 2003;63:1256–64.
30. Lin SL, Chen RH, Chen YM, et al. Pentoxifylline inhibits platelet derived growth factor-stimulated cyclin D1 expression in mesangial cell by blocking Akt membrane translocation. *Mol Pharmacol* 2003;64:811–22.
31. Chu TS, Wu MS, Hsieh BS. Vasodilator agents inhibit opossum kidney proximal tubular cell growth. *J Formos Med Assoc* 2005;104:374–7.
32. Hung KY, Chen CT, Yen CJ, et al. Dipyridamole inhibits PDGF-stimulated human peritoneal mesothelial cell proliferation. *Kidney Int* 2001;60:872–81.
33. Hocher B, Thone-Reineke C, Rohmeiss PP, et al. Endothelin-1 transgenic mice develop glomerulonephrosis, interstitial fibrosis, and renal cysts but not hypertension. *J Clin Invest* 1997;99:1380–9.

34. Benigni A, Zoja C, Corna D, et al. Blocking both type A and B receptors in the kidney attenuates renal injury and prolongs survival in rats with remnant kidney. *Am J Kidney Dis* 1996;27:416–23.
35. Benigni A. Endothelin antagonists in renal disease. *Kidney Int* 2000;57:1778–94.
36. Rich S, McLaughlin VV. Endothelin receptor blockers in cardiovascular disease. *Circulation* 2003;108: 2184–90.
37. Chu TS, Tsuganezawa H, Peng Y, et al. Role of tyrosine kinase pathways in ET_B receptor activation of NHE-3. *Am J Physiol-Cell Physiol* 1996;271:C763–71.
38. Amemiya M, Yamaji Y, Cano A, et al. Acid incubation increases NHE-3 mRNA abundance in OKP cells. *Am J Physiol-Cell Physiol* 1995;268:C126–33.
39. Tsuganezawa H, Sato S, Yamaji Y, et al. Role of c-SRC and ERK in acid induced activation of NHE3. *Kidney Int* 2002; 62:41–50.